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Combined nutritional stress and a new systemic pesticide (flupyradifurone, Sivanto®) reduce bee survival, food consumption, flight success, and thermoregulation

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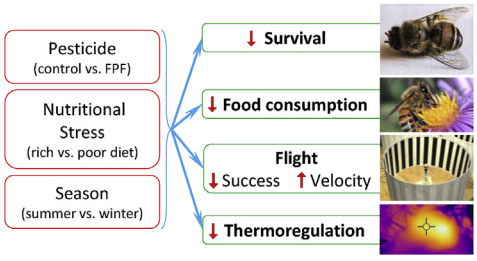
HIGHLIGHTS

- Flupyradifurone (FPF) significantly reduced bee survival in summer (−14%).
- Combined FPF and nutritional stress reduced post-flight thermoregulation (−1 °C, −4%).
- Interaction between FPF, nutrition, and season reduced bee food consumption (−14%).
- FPF, nutrition, season, and temperature alter flight (success: −19%; velocity: +13%).
- Nutritionally stressed bees cannot meet caloric needs (satiated at 54 ± 1 μl/bee/day).

GRAPHICAL ABSTRACT



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ABSTRACT

Flupyradifurone (FPF, Sivanto®) is a new butenolide insecticide that, like the neonicotinoids, is a systemic nicotinic acetylcholine receptor (nAChR) agonist. However, FPF is considered bee-safe (according to standard Risk Assessment tests), and is thus a potential solution to the adverse effects of other pesticides on beneficial insects. To date, no studies have examined the impact of nutritional stress (decreased food diversity and quality) and FPF exposure on bee health although both stressors can occur, especially around agricultural monocultures. We therefore tested the effects of a field-realistic FPF concentration (4 ppm, $FPF_{daily\ dose} = 241 \pm 4$ ng/bee/day, 1/12 of LD_{50}) and nutritional stress (nectar with low-sugar concentrations) on honey bee (*Apis mellifera* L.) mortality, food consumption, thermoregulation, flight success (unsuccessful vs. successful), and flight ability (duration, distance, velocity). Flight and thermoregulation are critical to colony health: bees fly to collect food and reproduce, and they thermoregulate to increase flight efficiency and to rear brood. We studied the effects across seasons because seasonality can influence bee sensitivity to environmental stress. We demonstrate that, depending upon season and nutritional stress, FPF can reduce bee survival (−14%), food consumption (−14%), thermoregulation (−4%, i.e. hypothermia), flight success (−19%), and increase flight velocity (+13%). Because

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pesticide exposure and nutritional stress can co-occur, we suggest that future studies and pesticide risk assessments consider both seasonality and nutritional stress when evaluating pesticide safety for bees.

1. Introduction

The honey bee, *Apis mellifera* L., provides crucial ecosystem services via pollination of native plants and crops worldwide (Potts et al., 2010). However, the health of managed honey bees has decreased globally (Lee et al., 2015; Potts et al., 2010). Large annual losses of managed honey bees are problematic given their role in pollinating crops and native plants, and because the costs of maintaining healthy bee stocks for agricultural pollination are increasing (Seitz et al., 2015). Recent declines in honey bee colonies may therefore impact crop production costs and perhaps even affect native ecosystems around the globe (Klein et al., 2007).

Factors contributing to recent bee losses include exposure to agricultural chemicals (Henry et al., 2015; Sanchez-Bayo, 2014), environmental variation causing malnutrition (Naug, 2009), and synergistic effects between these factors (Tosi et al., 2017b). Pesticides have received attention because they target pest insects (Jeschke and Nauen, 2008), but can harm beneficial insects. Honey bees may use pesticide-treated crops as a food source and are widely exposed to chemical residues (Tosi et al., 2018) drifting from crops (David et al., 2016), which persist in the environment after pesticide use has ceased (Sanchez-Bayo, 2014). In particular, one group of pesticides, the neonicotinoids, has been closely studied for their impact on honey bee health (Pisa et al., 2017; Sanchez-Bayo, 2014).

Neonicotinoids act upon the central nervous system of insects as agonists of the nicotinic acetylcholine receptors (nAChRs) and can cause lethal and sublethal effects in bees (Crall et al., 2019, 2018; Pisa et al., 2017; Tosi et al., 2017a, 2016; Tosi and Nieh, 2017). Bee flight ability (duration, distance, average velocity, and maximum velocity of flights) is altered by chronic or acute exposure (Tosi et al., 2017a). Blanken et al. (2015) showed that flight ability was reduced by *Varroa* infestation, and that this effect was stronger in the presence of the neonicotinoid imidacloprid. Neonicotinoids also alter thermoregulation in honey bees (Tosi et al., 2016) and bumble bees (Crall et al., 2018; Potts et al., 2018), impairing their ability to rewarm after thermal stress (a period of sustained chilling) or to regulate colony temperature. Neonicotinoids can also change bee energy levels (Tosi et al., 2017b) and food consumption (Kessler et al., 2015; Tosi et al., 2017b; Tosi and Nieh, 2017).

In response to the concerning effects of neonicotinoids on bees and growing pest resistance, a new generation of pesticides has been developed. Flupyradifurone (FPF), included in commercial formulations like Sivanto®, is a butenolide insecticide that is chemically similar to neonicotinoids (Giorio et al., 2017; Nauen et al., 2014). Like neonicotinoids, FPF is systemic and binds to nAChRs, but its bioactivation and structure−activity relationships differ from other nAChR agonists (Jeschke et al., 2015). FPF is effective against sucking pests that are resistant to neonicotinoids and is used for citrus, cocoa, cotton, grapes, hops, pome fruits, potatoes, soybeans, ornamental plants, and multiple other crops (Nauen et al., 2014).

FPF was reported to have no adverse effects on honey bees, allowing its application via spray on blooming crops with actively foraging bees (Nauen et al., 2014; US EPA, 2014). As part of the registration process for FPF, Risk Assessment (RA) experiments have tested the effects of FPF on honey bees (US EPA, 2014). However, pesticide RA procedures do not thoroughly test the sublethal effects of chemicals (Decourtye et al., 2013). Campbell et al. (2016) tested the effects of FPF and observed no significant side-effects on bee colony strength. However, in this latter study, bee-collected nectar and pollen from control fields were contaminated with FPF too, highlighting the difficulty of performing reliable ecotoxicological field trials (Simon-Delso et al., 2017).

Tosi et al. (2019) demonstrated that field-realistic worst-case FPF exposures cause sublethal and lethal synergistic effects in bees when combined with a common fungicide (propiconazole), and that FPF toxicity varied across season and bee age. Adverse effects on survival and abnormal behaviours began at a field-realistic dose of 375 ng FPF/bee; FPF was more toxic to foragers compared to in-hive bees, and more toxic to summer bees compared to early spring bees (Tosi and Nieh, 2019). Tan et al. (2015) demonstrated that chronic exposure to FPF impaired olfactory learning in larval (33 ng/larvae/day) and adult (66 ng/adult bee/day) Asian honey bees (*Apis cerana*). Hesselbach and Scheiner (2019, 2018) showed that acute exposure to a high, non-field realistic FPF dose (1200 ng/bee) impaired bee taste, cognition, and motor abilities. However, no studies have yet examined the sublethal effects of FPF on several other factors that influence bee health: food consumption, flight success (being able to fly), flight ability (detailed aspects of flight), and thermoregulation.

Flight is essential for pollination services and colony fitness because it allows bees to collect food, to protect the colony, and to reproduce. We therefore used a standard assay of bee flight ability: bees flying in flight mills (Tosi et al., 2017a). Honey bee flight ability depends upon flight muscle temperature (Esch, 1988, 1976; Schmaranzer, 2000; Schmaranzer and Stabentheiner, 1988), which is related to thoracic temperature (Woods et al., 2005). In addition, these flight muscles are a major source of heat production for nest thermoregulation (Bujok et al., 2002; Weidenmüller et al., 2002) and during recruitment (Stabentheiner et al., 1995; Stabentheiner and Hagmuller, 1991). Both flight (Tosi et al., 2017a) and heat production (Tosi et al., 2016) are altered by pesticides.

Nectar intake provides the energy required for thermoregulation (Gould and Gould, 1988), and thermoregulation and flight ability can depend on nectar sugar concentration (Gmeinbauer and Crailsheim, 1993). The quality of available nectar fluctuates greatly, typically from 5 to 80% (w/v) sugar concentration (Abrol, 2012; Crane, 1975), and nectar sugar concentrations can be as low as 2% (Abrol, 2012). In agricultural monocultures, decreasing floral diversity may limit the quality of nectar that bees can access (Donkersley et al., 2014; Naug, 2009) and, consequently, the energy available for flight and thermoregulation. Tosi et al. (2017b) demonstrated that consuming low sucrose nectar containing sublethal field-realistic doses of pesticides could cause adverse synergistic effects on bees by reducing survival, glucose and trehalose hemolymph concentrations, and food consumption.

Seasonality can also influence bee sensitivity to environmental stressors, such as pesticides and nutritional stress (Poquet et al., 2016; Tosi and Nieh, 2019). Tosi et al. (2019) demonstrated that FPF is more toxic in summer as compared to early spring. Hesselbach and Scheiner (2019) also observed variations in the

effects of FPF depending on the season the bees were collected. Honey bees can adapt to seasonal changes and food scarcity by modifying their foraging range (Schneider and McNally, 1993) and recruitment strategies (Park and Nieh, 2017). Bees are differentially affected by pesticides according to season: following pesticide treatment, winter bees showed higher mortality than summer bees (Decourtye et al., 2003). We therefore considered the effects of two seasons, winter (September to February) and summer (March to August).

Our work aimed at providing further insights on the complex and subtle effects that pesticides could have on bee behaviour and health. Therefore, we studied the individual and combined effects of FPF, nutrition (*rich* vs. *poor* quality diets), and season on survival, thermoregulation (measured as thoracic surface temperature), food consumption, flight success, and flight ability.

2. Methods

This study was conducted from 2016 to 2017 at the University of California, San Diego (UCSD). We tested 1276 bees from ten healthy honey bee colonies (*A. mellifera ligustica* Spinola, 1806, 10 frames per colony) housed at the UCSD Biology Field Station apiary. We collected foragers that were subsequently exposed to a nutritional stress (*ad libitum* access to nectar with low sugar concentration) and a pesticide treatment (FPF) across two seasons (winter and summer) using a full factorial design. We applied standard inspection techniques (Dietemann et al., 2013; Higes et al., 2011) to confirm that our colonies were healthy and did not have detectable disease or parasite infestations.

2.1. Honey bee preparation

We collected returning pollen foragers at their hive entrances, identified as returning bees carrying pollen loads (Henry et al., 2015; Tosi et al., 2017a). The collected foragers were transferred into cages (10 individuals per cage) incubated at $30 \pm 1\,^{\circ}C$ and 50–80% RH for 3 days, and provided *ad libitum* sucrose solution through a 5 ml syringe. All bees were chronically exposed to the sucrose solution to simulate exposure over multiple days. The sucrose and pesticide content of the solution varied depending on the sugar diet and FPF treatments (see Methods below), simulating foraging on contaminated fields that produce nectar of poor nutritional quality (lower sugar concentration). Each 24 h, we measured survival and food consumption. After 3 days of incubation, we tested bee thermoregulation and flight. We measured survival once per day, after the thermoregulation and flight measurements, until bee death. Food consumption was measured for bees that were tested in flight mills. Thermoregulation was measured with a randomly selected subset of flown bees.

2.2. Sugar diet treatments

In our study, we tested a nutritional stress scenario of limited carbohydrate intake, feeding bees a diet with reduced sucrose concentration (the *poor* diet). We fed bees an *ad libitum* sugar diet of either *rich* (50% w/w sucrose solution) or *poor* (33%, leading to a nutritional stress) quality (Crane, 1975; Tosi et al., 2017b). The diet was either pure sucrose solution (control) or contained FPF, depending on the assigned treatment. These nutritional treatments were field-realistic, because foragers can intake these sugar concentrations when ingesting nectar (5–80%, Abrol, 2012; Crane, 1975) or non-ripened honey stored in the nest (Atkins et al., 1975; Crane, 1975). In addition, nutritional stress can also be caused by non-foraging periods. Insufficient food stores are a common cause of winter colony losses (Brodschneider and Crailsheim, 2010; Seitz et al., 2015). More details about the field-realism of the nutritional treatments can be found in Tosi et al. (2017b).

2.3. FPF treatment

We followed the most recent international guidelines for pesticide tests on bees (OECD/OCDE, 2017, 1998). Because FPF is a relatively recent pesticide, there is still limited environmental contamination data available (Campbell et al., 2016; US EPA, 2014). However, concentrations of 4.3 ppm (4300 μg/kg) and 4.1 ppm (4108 μg/kg) of FPF were found in the honey stomach of foragers collecting nectar from oilseed rape fields treated with the recommended FPF concentration in France and Northern Germany (US EPA, 2014).

We calculated the worst-case field-realistic FPF oral exposure level for bees following European Food Safety Authority (EFSA) and Environmental Protection Agency (EPA) methods. Foragers collecting nectar in a field previously sprayed with FPF can intake up to 5504 ng FPF/bee per foraging day. According to other calculations (US EPA, 2012), the refined Estimated Environmental Concentration (EEC) of FPF is 1256 ng/forager for oilseed rape crops (US EPA, 2014). When bees forage nectar in cotton fields, refined EEC for workers reaches 6370 ng FPF/bee (US EPA, 2014).

We tested sublethal acute oral exposure to field-realistic concentration and daily doses of FPF (4 μg/kg, corresponding to 4 ppm; $FPF_{daily\ dose} = 241 \pm 4$ ng/bee/day). This daily dose was 12.4 times lower than the LD_{50} of FPF (2995 ng/bee, Tosi and Nieh, 2019). The LD_{50} of FPF calculated during the study period with bees from our study apiary (Tosi and Nieh, 2019) was higher than that reported by US EPA (2014). Similar LD_{50} variation has been observed for other agonists of insect nAChRs (IRAC Group 4), including the neonicotinoids (EFSA, 2012; Pisa et al., 2014). Our FPF concentration and daily dose were thus field-realistic because bees can consume higher concentrations and daily doses of FPF by ingesting contaminated nectar in the field.

Because FPF has a wide spectrum of pest targets and application methods, it can be used across different seasons (Nauen et al., 2014) for agricultural crops and ornamental plants (Nauen et al., 2014) that flower at different times throughout the year, leading to long term exposure. FPF was found in the honey and nectar stored in bee combs for up to five months, and in the nectar collected by foragers for more than two weeks (winter oilseed rape fields, US EPA, 2014).

We chronically exposed our bees to FPF for three days. This duration was field-realistic because bees can be exposed to FPF for longer periods in the field (see above). All bees consumed $FPF_{daily\ doses}$ that were lower than the dose bees could consume in the field, in part because of the reduced energy requirement of bees confined in cages. This led to low daily consumption doses of sucrose ($Sucrose_{daily\ doses,\ rich\ nutrition} = 28.7 \pm 0.4$ mg/bee/day, $Sucrose_{daily\ doses,\ poor\ nutrition} = 22.1 \pm 0.3$ mg/bee/day, calculated on bees tested for flight and thermoregulation) and FPF ($FPF_{daily\ doses,\ rich\ nutrition} = 213 \pm 3$ ng/bee/day, $FPF_{daily\ doses,\ poor\ nutrition} = 266 \pm 6$ ng/bee/day).

Because of the limited amount of data on field-realistic FPF residues, our estimates were based on *ad hoc* trials performed for pesticide registration purposes, before product authorization. The estimates of FPF field-realistic doses and concentrations should be updated with more real-world data from multiple scenarios.

We used analytical grade FPF (Sigma Aldrich, CAS# 951659-40-8, catalog# 37050-100 MG) to create our pesticide treatment solutions. Solutions were freshly prepared each week in 50 ml tubes with double-distilled water. The tubes were stored at 4 °C in a dark refrigerator and tightly wrapped in aluminum foil to prevent light degradation.

2.4. Seasonality

Season influences bee sensitivity to pesticides (Tosi and Nieh, 2019), and foragers can be exposed to nutritional stress and FPF during different times of the year. We therefore tested field-realistic situations exposing bees to these environmental stresses throughout the year. We categorized our study period into two seasons, winter (September to February) and summer (March to August), to respectively reflect the cool and wet dormant season, and warm and dry growth season that are relevant to bees in our local ecosystem (Park and Nieh, 2017).

2.5. Survival: before and after flights

Bee survival was measured every 24 h during the 3-day incubation before and after flight testing. After the flight tests, bees were placed in individual cages and fed their respective treatment solutions *ad libitum* until death. A bee was considered dead when it was immobile and did not react to any stimulation (Medrzycki et al., 2013).

2.6. Food consumption

During the 3-day incubation, we calculated the weight of sucrose and FPF consumed per cage each day, and subsequently calculated the average amount of sucrose and FPF consumed per living bee. In the sugar consumption measurements, we factored the sucrose concentration of the sucrose solutions (50% or 33%), the density of the sucrose solution ($\delta_{50\%\ w/w} = 1229.65\ kg/m^3$, $\delta_{33\%\ w/w} = 1141.51\ kg/m^3$) (Bubnik et al., 1995), the number of live bees per cage per day, and the evaporation rate (<1%). To measure the average loss of solution due to evaporation, we kept cages with sucrose solution, but without bees, at the same incubator conditions.

2.7. Flight success and flight ability

The flight mills used were described in Tosi et al. (2017), and were based upon the designs of Smith and Jones (2012). Each flight mill consisted of a magnetically levitated, balanced arm upon which the bee flew while surrounded by a white paper cylinder with alternating black and white stripes to provide consistent optic flow. We harnessed each bee as described in Tosi et al. (2017). After harnessing, we rested bees by incubating them individually for 30 min (30 ± 1 °C and 50–80% RH) before testing them on flight mills. We recorded whether bees completed a successful flight or were not able to fly even after 10 min of repeated stimulation. Bees that successfully flew were monitored until exhaustion on the flight mills. For each bee, we used the longest continuous flight during their time in the flight apparatus to calculate flight duration, distance, and velocity ("flight ability"). Each bee was flown only once.

2.8. Thermoregulation

We measured the thoracic surface temperatures of the bees after 3 days of chronic exposure, before and after their flight with an imaging infrared thermography camera (Raytek High-Performance Thermal Imager, ThermoView Ti30, Fluke Process Instruments, Everett, Washington, USA). Infrared thermography is a standard, non-invasive method for measuring bee thoracic surface temperature to estimate honey bee thoracic muscle temperature in ecotoxicological trials (Tosi et al., 2016). Measured temperatures were calibrated with a known temperature source as described in Nieh et al. (2006).

2.9. Statistical analysis

We used Fit Proportional Hazards models to test the effect of $FPF_{daily\ dose}$, nutritional stress, season, their interactions, and colony identity upon bee survival before and after flight test. Significant effects were further analysed with Kaplan-Meier survival analysis (Wilcoxon Chi-square values).

Nominal Logistic Regression was used to test the effect of FPF, nutritional stress, season, their interactions, and colony on flight success. Significant effects were further analysed with Fisher's Exact test: 2 × 2, two-tailed, Pearson Chi-square values (Lowry, 2016).

We used Mixed Models (REML algorithm) to test the effects of FPF, nutritional stress, season, and their interactions on food consumption and thermoregulation ability. Colony was used as a random grouping variable. Based upon visual data inspection, effects were further analysed with post-hoc Least-Square Means contrast tests.

Mixed-Model Analysis of Covariance (ANCOVA, REML algorithm) was used to test the effects of FPF, nutritional stress, season, thorax $temperature_{before\ flight}$, and their interactions on flight ability: duration, distance, average velocity, maximum velocity. Distance and duration were log-transformed to normalize the data. The mixed model allowed testing for both nominal (FPF, nutritional stress, season) and continuous (thorax $temperature_{before\ flight}$) variables. Colony was used as random variable. We used linear regression to further analyze the significant effects of thorax $temperature_{before\ flight}$ on bee flight ability, computing separate analysis depending on FPF, nutritional stress, and season.

We used JMP Pro v14.0.0 statistical software and applied residuals analyses to confirm the appropriateness of our models. Based upon visual data inspection, effects were further analysed with post-hoc Least-Square Means contrast tests, as appropriate. We used an alpha value of 0.05, but applied the Dunn-Sidak method (Sokal and Rohlf, 1995), as appropriate, to correct for multiple comparisons and indicate tests that pass with ^{DS}. We used stepwise model simplification (Crawley, 2012). We report mean ± 1 standard error (s.e.m.). We provide a negative percentage when reporting a percentage decrease and positive percentage to indicate an increase.

3. Results

3.1. FPF reduced bee survival

As expected, bees had higher survival rates when offered higher quality nutrition ($p < 0.0001$, Table 1). There was a significant effect of season: summer bees survived being caged longer than winter bees ($p = 0.012$). Although there was also no significant effect of $FPF_{daily\ dose}$ alone on survival ($p = 0.16$), the interaction FPF_{daily}

Table 1
Summary of the effects of FPF, nutritional stress, and season on bee survival before (during the 3 days incubation) and after the flight tests (Fit proportional hazard test, $N_{before\ tests} = 1276$; $N_{after\ tests} = 338$). See Fig. 1 for a graph of the significant results.

Period	Factor	DF	L-R χ^2	*P*-value
Before flight	$FPF_{daily\ dose}$	1	2.00	0.157
	Nutritional stress	1	90.42	**<0.001**
	Season	1	6.27	**0.012**
	$FPF_{daily\ dose}$ × Season	1	6.12	**0.013**
	Colony	10	103.08	**<0.0001**
After flight	$FPF_{daily\ dose}$	1	1.30	0.255
	Nutritional stress	1	2.85	0.092
	Season	1	2.63	0.105
	Colony	10	40.24	**<0.0001**

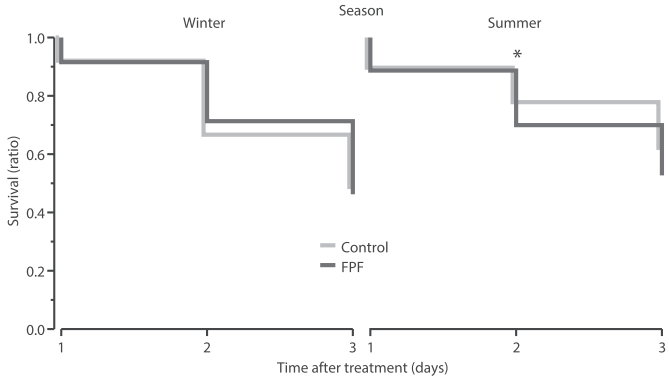

Fig. 1. Effects $FPF_{daily\ dose}$ on bee survival before flight (during the 3-day incubation) in Winter (left) and Summer (right). The lines are slightly shifted to better display survival trends. More details are reported in Table 1. The asterisk indicates a significant effect (Fit proportional hazards, Kaplan-Meier, $^{*}p < 0.05$).

$_{dose}$ × season was significant ($p = 0.013$). $FPF_{daily\ dose}$ reduced bee survival in summer (−14%, Kaplan-Meier, DF = 1, $\chi^2 = 4.46$, $p = 0.035$, Fig. 1). The significant effects occurred after bee collection, during the chronic exposure to FPF before flight. No significant effects on bee survival were found after flight.

3.2. Combined FPF and nutritional stresses reduced food consumption

There was a main significant effect of FPF, nutritional stress, season, and all interactions ($p < 0.037$, Table 2) on bee food consumption. As expected, nutritional stress reduced the daily consumption of sucrose (−23%, $Sucrose_{daily\ doses,\ rich\ nutrition} = 28.7 \pm 0.4$ mg/bee/day, $Sucrose_{daily\ doses,\ poor\ nutrition} = 22.1 \pm 0.3$ mg/bee/day, $p < 0.0001$), and winter bees, characterized by higher energy stores (Ribbands, 1953; Winston, 1987), consumed less sucrose than summer bees (−9%, $p = 0.001$). FPF significantly reduced sucrose consumption ($p < 0.001$). Specifically, FPF reduced food consumption of summer (but not winter) bees fed high quality nutrition (−14%, contrast test, $F_{1,327} = 497.63$, $p < 0.0001$, Fig. 2A).

As expected, the nutritional treatment altered the consumption of sucrose and FPF, such that bees fed the *poor* diet (33% sucrose w/w) ingested a lower mass of sucrose (−23%, mass of $sucrose_{daily\ doses,\ poor\ nutrition} = 22.1 \pm 0.3$ mg/bee/day, Fig. 2A and Table 2), a greater volume of sucrose solution (+15%, volume of sucrose $solution_{poor\ nutrition} = 54.4 \pm 0.8$ µl/bee/day), and thus a greater dose of FPF (+20%, $FPF_{daily\ doses,\ poor\ nutrition} = 266 \pm 5$ ng/bee/day) than bees on the rich diet.

3.3. Combined FPF and nutritional stresses decreased flight success of winter bees

There were no significant effects of FPF, nutritional stress, or season alone upon flight success (Table 3). However, there was a significant three-way interaction between FPF × nutritional stress × season ($p = 0.014$). FPF reduced the flight success of nutritionally stressed winter bees (−19%, Fisher Exact test, $\chi^2 = 7.93$, $p = 0.008$, Fig. 2B).

Table 2
Summary of the effects of FPF, nutritional stress, and season on bee food consumption (sucrose weight intake) over 3 days of incubation (top, Fig. 2A), flight ability (middle, Fig. 2C), and thorax temperature (bottom, before and after flight, Fig. 2D). The REML variance component estimates of colony effect are 55% for bee food consumption (Mixed $Model_{REML}$, $N = 338$), ≤1% for flight ability (Mixed-Model $ANCOVA_{REML}$, $N = 209$), and 41% and 73% for thorax temperature respectively before and after flight (Mixed $Model_{REML}$, $N = 234$).

Measurement	Factor	DF numerator	DF denominator	*F* ratio	*P*-value
Food consumption	FPF	1	321	14.13	**<0.001**
	Nutritional stress	1	325	171.22	**<0.0001**
	Season	1	328	6.78	**0.010**
	FPF × Nutritional stress	1	323	4.38	**0.037**
	FPF × Season	1	324	13.52	**<0.001**
	Nutritional stress × Season	1	326	12.31	**0.001**
	FPF × Nutritional stress × Season	1	324	7.71	**0.006**
Flight ability:Average velocity	FPF	1	203	1.50	0.221
	Nutritional stress	1	190	1.47	0.227
	$Thorax\ temperature_{before\ flight}$	1	50	9.18	**0.004**
	Season	1	46	2.19	0.146
	FPF × Season × $Thorax\ temperature_{before\ flight}$	1	202	4.69	**0.032**
Flight ability:Maximum velocity	FPF	1	202	1.97	0.162
	Nutritional stress	1	192	1.45	0.230
	$Thorax\ temperature_{before\ flight}$	1	47	11.58	**0.001**
	Season	1	48	4.15	**0.047**
	FPF × Nutritional stress × Season	1	180	4.75	**0.031**
	FPF × Season × $Thorax\ temperature_{before\ flight}$	1	200	6.24	**0.013**
Flight ability:Duration	FPF	1	204	2.69	0.102
	Nutritional stress	1	196	1.05	0.306
	$Thorax\ temperature_{before\ flight}$	1	47	1.66	0.204
	Season	1	47	0.51	0.477
Flight ability:Distance	FPF	1	204	2.48	0.117
	Nutritional stress	1	196	1.25	0.265
	$Thorax\ temperature_{before\ flight}$	1	41	2.42	0.127
	Season	1	42	0.81	0.375
Thorax temperature *before* flight	FPF	1	226	0.04	0.836
	Nutritional stress	1	229	0.00	0.980
	Season	1	230	28.07	**< 0.0001**
Thorax temperature *after* flight	FPF	1	223	1.88	0.172
	Nutritional stress	1	224	10.50	**0.001**
	Season	1	225	87.17	**< 0.0001**
	FPF × Nutritional stress	1	224	3.87	0.051

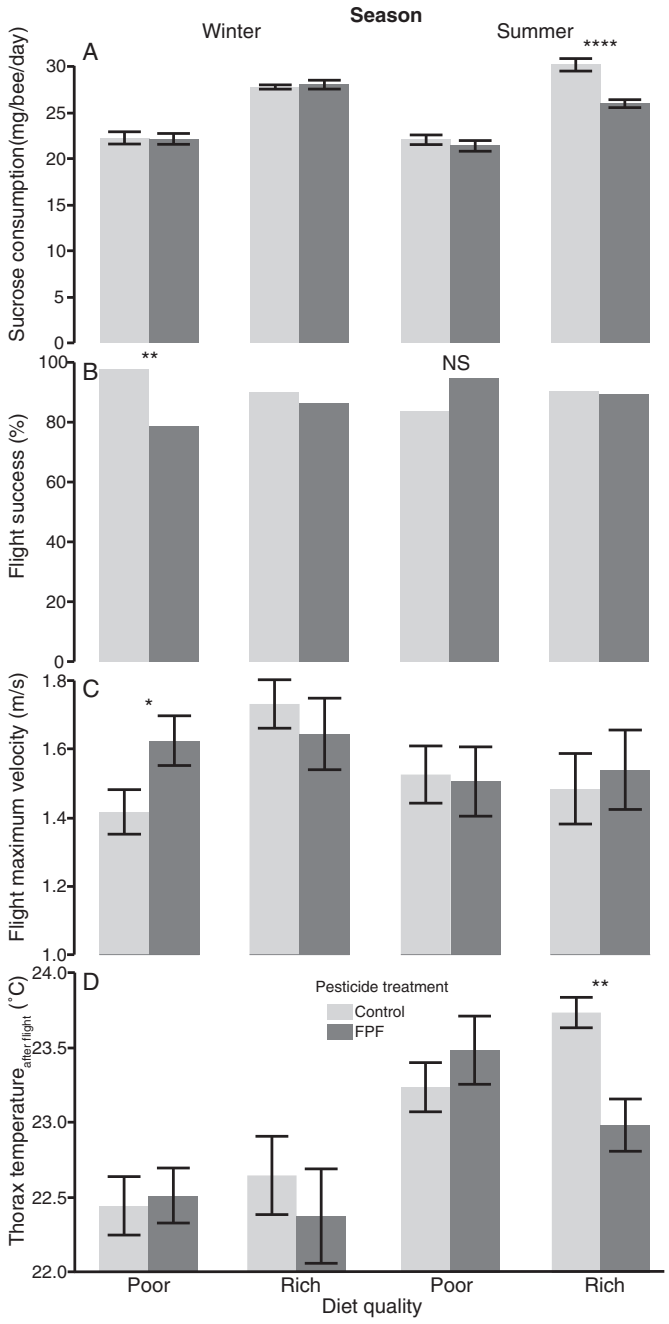

Fig. 2. Effect of FPF (4 ppm) and diet quality (poor: 33% sucrose concentration, rich: 50% sucrose concentration) on bee (A) food consumption, (B) flight success, (C) maximum flight velocity, and (D) thorax temperature$_{\text{after flight}}$, in Winter (left) and Summer (right). More details are reported in Table 2. The asterisks indicate a significant effect (A, B: nominal logistic, Fisher exact test^{DS}, $^{**}p < 0.01$; C, D: Mixed Model ANCOVA$_{REML}$, contrast test, $^{*}p < 0.05$; E, F: Mixed Model$_{REML}$, contrast test; G, H: Mixed Model$_{REML}$, contrast test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$, NS = Not Significant). Error bars show standard errors.

3.4. Combined FPF and nutritional stresses altered flight ability depending on season

There were no significant effects of FPF or nutritional stress on flight ability ($p > 0.10$, Table 2). There was only a significant effect of season on maximum velocity ($p = 0.047$). However, the interaction of FPF × nutritional stress × season did significantly influence flight maximum velocity ($p = 0.031$). Specifically, FPF significantly increased the maximum flight velocity (+13%) of winter bees fed the lower quality diet, as compared to control winter bees fed the same diet (contrast test, $F_{1,192} = 7.13$, $p = 0.008$, Fig. 2C).

Table 3
Summary of the effects of FPF, nutritional stress, and season on bee flight success (Nominal Logistic regression, $N = 338$). See Fig. 2B for a graph of the significant results.

Factor	DF	L-R χ^2	*P*-value
FPF	1	0.57	0.452
Nutritional stress	1	0.39	0.534
Season	1	0.66	0.416
FPF × Nutritional stress × Season	1	6.05	**0.014**
Colony	10	10.87	0.368

Because flight power is directly related to flight muscle temperature, we tested the impact of thorax temperature on flight ability. There was a significant effect of thorax temperature$_{\text{before flight}}$ upon flight average ($p = 0039$) and maximum ($p < 0.001$) velocity (Table 2). The interaction of FPF × season × thorax temperature$_{\text{before flight}}$ significantly influenced average ($p = 0.032$) and maximum ($p = 0.013$) flight velocities (Table 2). There was also a significant positive correlation between thorax temperature$_{\text{before flight}}$ and average and maximum flight velocity when bees were fed pesticide-free diets of *rich* quality during the summer ($R^2 > 0.29$, $F_{1,15} \leq 6.64$, $p \leq 0.025$). Further analyses demonstrated that maximum and average flight velocity correlated positively with thorax temperature$_{\text{before flight}}$ when bees were exposed to both stressors (FPF and nutritional deficiency) in winter ($R^2 \geq 0.25$, $F_{1,34} \leq 12.13$, $p \leq 0.002$).

3.5. Combined FPF and nutritional stresses reduced thorax temperature depending on season

Summer bees had significantly higher thorax temperatures before and after flight ($p < 0.0001$, Table 2). Before flight, there were no significant effects of nutrition ($p = 0.98$) or FPF ($p = 0.84$) on bee thorax temperature (Table 2). After flight, there was a significant effect of nutrition ($p = 0.001$) on bee thorax temperature such that bees fed the *rich* diet had a higher thorax temperature than bees fed the *poor* diet (Table 2). There was no significant effect of FPF alone ($p = 0.17$). However, there was a significant combined effect of FPF and nutritional treatment on bee thorax temperature after flight: specifically, FPF reduced the thorax temperature after flight of summer bees fed the *rich* diet, as compared to control summer bees fed the same diet (−4%, −1 °C, contrast test, $F_{1,221} = 6.90$, $p = 0.009$, Fig. 2D).

4. Discussion

We demonstrate, for the first time, that field-realistic nutritional stress and FPF can, individually and in combination, impair bee health through lethal and sublethal effects. These effects are influenced by season. Our work highlights how the effects of pesticides can be subtle and are sometimes only revealed as an interaction with other factors, such as nutritional status, season, and flight exertion.

FPF reduced bee thermoregulation ability after flight (−1 °C, -4%, Table 2, Fig. 2D), a high intensity task (Beenakkers et al., 1984). Before flight, there was no effect of nutritional stress or FPF on bee thorax temperature, confirming the low sublethal levels of the tested treatments. However, after summer flights, the combination of FPF and nutritional treatment did alter bee thorax temperature. FPF reduced the thorax temperature after flight of summer bees fed the rich diet, compared to pesticide-free bees exposed to the rich

diet (Fig. 2D). We hypothesize that the interaction of rich diet and FPF have elicited behavioural and physiological responses in the short-term (i.e. increased thermogenesis, motor activity, hyperactivity; Potts et al., 2018; Tosi et al., 2016; Tosi and Nieh, 2019) that led to higher exertion and energy exploitation as compared to the poor nutritional treatment, which caused hypothermia (lower body temperature) in the longer-term, after flight (Fig. 2D). We speculate that the nutritionally deficient *poor* diet may have halted the increased activity elicited in summer bees fed the *rich* diet and FPF.

Like the neonicotinoids (Tosi et al., 2016), FPF exposure may have increased bee energy requirements (Tosi et al., 2017b), perhaps due to detoxification demands, to changes in bee energy metabolism (du Rand et al., 2017), or both. Although FPF may increase energy consumption, FPF-treated bees did not increase their sucrose consumption, similar to the results found for bees exposed to neonicotinoids (Kessler et al., 2015; Tosi and Nieh, 2017). It is unclear why this is the case, but these pesticides may have broad effects given that they target a common receptor found in multiple neuron types and influence multiple behaviours such as feeding. The extremely energy-intensive behaviour of bees flying to exhaustion likely revealed the subtle combined effect of pesticide and nutritional stress upon bee thermoregulation after flight.

This impairment may have other consequences. Flight muscles are in the thorax and are a major source of shivering thermogenesis in bees (Heinrich and Esch, 1994; Roberts and Harrison, 1998). Thus, the reduction of bee thermoregulation ability after flight can impair colony fitness because bees need to thermoregulate while unloading their collected food or waggle dancing to recruit nestmates after returning to the colony (Stabentheiner et al., 1995; Stabentheiner and Hagmuller, 1991).

Field-realistic exposure to FPF reduced forager survival. These effects were influenced by season: FPF reduced bee survival in summer (−14%, Table 1, Fig. 1), confirming a prior study showing that FPF toxicity increases in summer (Tosi and Nieh, 2019).

FPF reduced food consumption (−14%) of bees reared at optimal conditions (*rich* nutrition, summer, Fig. 2A). Bees that were fed *poor* nutrition consumed a greater volume of solution (+15%, perhaps because of hunger), thus increasing their consumption of FPF (+20%; mean and s.e.m.: 266 ± 5 ng/bee), as compared to bees fed *rich* nutrition. Consequently, in field-realistic scenarios of pesticide contamination, bees that are malnourished or exposed to low-quality nutrition could face an amplified risk due to increased pesticide exposure. This scenario is concerning, given that pesticides and nutritional stress have adverse synergistic effects on bees (Tosi et al., 2017b).

Nutritionally stressed bees became satiated before daily caloric needs were met. Although the nutritional stress increased the volume of sucrose solution consumed as expected (+15%, sucrose solution volume$_{poor\ \text{nutrition}} = 54.4 \pm 0.8$ µl/bee/day), sucrose intake was still lower (−23%, sucrose weight$_{rich\ \text{nutrition}} = 28.7 \pm 0.4$ mg/bee/day), as compared to bees fed the *rich* diet ($p < 0.0001$, Table 2, Fig. 2A). These findings support previous results, demonstrating that sucrose solution satiation occurs at 64 ± 1 µl/bee/day (Tosi et al., 2017b). We also showed that winter bees, characterized by higher energy stores (Mattila et al., 2001; Ribbands, 1953), required less sucrose than summer bees ($p = 0.010$).

The combined field-realistic exposure to FPF and nutritional stress reduced flight success in winter bees (−19%, Table 2, Fig. 2B). The rich nutrition seemed to buffer the effect of FPF on flight success. Conversely, the poor nutrition diet was not enough to protect winter bees from the adverse effects of FPF, perhaps because nutritional stress and pesticides act synergistically to reduce bee health and energy levels (Tosi et al., 2017b), and toxin detoxification requires energy (du Rand et al., 2017). Foraging flights are essential to collect food for the hive (Riley et al., 2005), and impairing flight success should reduce colony fitness.

Flight ability was altered by combined exposure to FPF and nutritional stress, and these effects were influenced by season and bee body temperature before flight (Table 2, Fig. 2C). The increased maximum velocity of flights (+13%) caused by FPF may be a kind of hyperactivity, a typical short-term effect of FPF (Tosi and Nieh, 2019) and nicotinic acetylcholine receptor (nAChRs) agonists (Gill and Raine, 2014; Tosi et al., 2017a; Tosi and Nieh, 2017). This alteration was only significant in winter bees exposed to a concomitant nutritional stress.

Chronic nutritional stress alone did not significantly influence flight ability ($p \geq 0.23$). Carbohydrate concentration (1–4 M glucose, feeding a single dose) is known to positively correlate with the speed of bees in flight mills (Balderrama et al., 1992; Gmeinbauer and Crailsheim, 1993). Because we fed bees continuously over multiple days, they may have built up their flight reserves, buffering the effects of our nutritional stress. Another possibility is that nutritional stress in conjunction with pesticide exposure reduced bee survival before flight, leaving only the more resistant and healthy bees for flight testing.

Winter and summer bees differ in multiple ways. Summer bees usually spend more time flying and less time thermoregulating as compared to winter bees (Mattila et al., 2001; Rortais et al., 2005). Summer bees also have reduced energy stores, longevity, and are less resistant to multiple stressors (Ribbands, 1953; Winston, 1987) including pesticides (Decourtye et al., 2003; Tosi and Nieh, 2019). We found that FPF reduced survival, food consumption, and thermoregulation of summer bees, which have less robust survival, food stores, and thermoregulatory abilities than winter ones (Mattila et al., 2001). Similarly, FPF reduced flight success and altered flight ability in winter bees, which may fly less, as compared to summer bees (Mattila et al., 2001; Rortais et al., 2005). These results support prior data demonstrating that FPF toxicity changes across season (Tosi and Nieh, 2019).

We also show that bees with warmer flight muscles flew faster, as expected (Table 2). These higher thoracic temperatures should increase the ability of bees to fly and thereby to retrieve food (Woods et al., 2005). We captured these effects at different situations, when bees were exposed to both good (summer pesticide-free foragers fed higher sugar content diets) and sub-optimal (winter bees fed lower sugar content diets with pesticide) conditions. Because our bees were not the exact same age, this may have affected our experimental outcomes such as increasing variance in our measured effects. However, all tested bees belonged to the foraging caste and were therefore in the same age group, and studying foragers as an overall group has relevance for understanding the real-world impact of pesticides.

As with other relatively new pesticides, the contamination levels of FPF in the environment are largely unknown, especially for winter bees. Further screening of environmental contamination following real-world use of pesticides over a broad spectrum of environmental conditions (including across seasons) is crucial for appropriately assessing actual residue levels and consequent pesticide risk. Nonetheless, independently from the estimation of field-realism, our work demonstrates how pesticide toxicity varies depending on multiple environmental factors.

Our results align with recent research showing that FPF, examined as a single factor, has little or no adverse effects on honey bees (Campbell et al., 2016; Hesselbach and Scheiner, 2019, 2018; Tosi and Nieh, 2019). However, field-realistic levels of FPF can synergistically impair *A. mellifera* survival and behaviour when combined with another pesticide, and FPF toxicity is significantly influenced by season and worker type (Tosi and Nieh, 2019). Field-realistic FPF exposure alone can also impair cognition in an Asian honey bee species, *Apis cerana* (Tan et al., 2015). Tosi et al. (2017b) also

demonstrate that pesticides interact with non-pesticide field-realistic stressors, such as nutritional stress (e.g. starvation), to alter bee health. When seasonality and nutritional stress were examined, our study found that FPF altered bee survival, food consumption, flight, and thermoregulation. We thus provide further insights on the complex and subtle effects that pesticides elicit on bee behaviour. These sublethal effects may impair colony health. Future studies – in the lab and in the field – should therefore holistically examine multiple factors and bee behaviours, and consider the role that seasonality and nutritional stress play in pesticide toxicity.

Conflicts of interest

The authors declare no competing financial interests.

Author contributions

ST and JCN conceived the experiments. ST, LT, and JCN designed the experiments. LT performed the experiments, collected and managed all data. JCN provided materials and reagents. ST, LT, and JCN analysed the data. ST, LT, and JCN wrote and reviewed the manuscript.

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Appendix A. Supplementary data

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